# Early Journal Content on JSTOR, Free to Anyone in the World

This article is one of nearly 500,000 scholarly works digitized and made freely available to everyone in the world by JSTOR.

Known as the Early Journal Content, this set of works include research articles, news, letters, and other writings published in more than 200 of the oldest leading academic journals. The works date from the mid-seventeenth to the early twentieth centuries.

We encourage people to read and share the Early Journal Content openly and to tell others that this resource exists. People may post this content online or redistribute in any way for non-commercial purposes.

Read more about Early Journal Content at http://about.jstor.org/participate-jstor/individuals/early-journal-content.

JSTOR is a digital library of academic journals, books, and primary source objects. JSTOR helps people discover, use, and build upon a wide range of content through a powerful research and teaching platform, and preserves this content for future generations. JSTOR is part of ITHAKA, a not-for-profit organization that also includes Ithaka S+R and Portico. For more information about JSTOR, please contact support@jstor.org.

# SOME CHEMICAL CONDITIONS INFLUENCING ACID-PROOFNESS AND NON-ACID-PROOFNESS IN A SAPROPHYTIC CULTURE OF B. TUBERCULOSIS.*

W. B. WHERRY.

*(From the Laboratory of the Cincinnati Hospital, Cincinnati, Ohio.)*

As has been already detailed by me,[1] a saprophytic culture of *B. tuberculosis*, when grown for a few days on such media as nutrient agar, glycerin agar, or glucose agar, was composed chiefly of bacilli which gave up the Ziehl-Nielson stain when treated with 10 per cent HCl in 95 per cent alcohol. However, in young cultures a few strongly acid-proof rods could always be found, and these increased in numbers with the age of the culture—especially in those on glycerin and glucose agar. The thick, yellow, wrinkled pellicle on the surface of +1 glycerin broth cultures grown for 4 to 6 weeks at 37° is entirely composed of acid-proof curved or straight rods imbedded in a tough matrix of unknown composition.

This extremely saprophytic culture was brought from R. Koch's laboratory, by Dr. V. C. Vaughan, in 1888. Most of the previous writers, excepting Sander, do not give the source or degree of saprophytism of their cultures. Apparently they were of a less saprophytic type and were still pathogenic. Sander's culture was of human origin and had been cultivated for about a month and a half.

While scattered observations on the occurrence of non-acid-proof bacilli in tubercle cultures have been made, it is only necessary to refer here to the observations of Tint, and Breskman[2] who, from a tinctorial study of old (16 years) and more recently isolated human cultures grown on glycerin agar, concluded that the acidfast property of cultures increases with the duration of growth and that non-acid-proof bacilli occur in greatest numbers in young cultures, though they may also be found in old cultures.

---

* Received for publication May 20, 1913.

[1] *The Lancet Clinic* (Cincinnati), 1913, 109, p. 134.

[2] *Jour. Am. Med. Assn.*, 1910, 54, p. 1591.

According to Chabas, Ferran[1] isolated a non-acid-proof strain of *B. tuberculosis* from an inoculated guinea-pig and was the first to show that a saprophytic strain could originate from a parasitic one.

During a study of the chemical requirements for the nutrition of my culture, notes were kept not only on the presence or absence of growth but on the morphologic and tinctorial characters of the bacilli, in the hope that a less toxic strain with some powers of immunization might result from a procedure which may be termed chemical selection.

While the studies of Sander,[2] Kuhne,[3] Uschinsky,[4] Proskauer and Beck,[5] and the more recent work of Tiffeneau and Marie,[6] Sauton,[7] and Armand-Delille[8] and his coworkers, have shown that the tubercle bacillus may grow luxuriantly in media of simple and known initial composition, it may be stated that the writer was unaware of most of these experiments, and hence the chemical conditions detailed below are not, in most instances, directly comparable with those found in previous work.

Further, these studies bring out points which apparently have not been noted before, e.g., some factors involved in the loss and acquisition of the acid-proof quality, and the influence of phosphates on the fermentation of carbohydrates.

The cultivations were carried on at 37° in 50 c.c. of various synthetic media placed in 500 c.c. narrow-mouthed bottles plugged with cotton. The media were sterilized in the autoclav or, when carbohydrates were present, at 100° on three successive days. When substances of a low boiling-point were to be added, this was done with a sterile pipette after the rest of the medium had been sterilized and cooled.

Some experiments, where acetates were the source of N. and C., showed that the area of exposed surface and volume of liquid apparently played no rôle in determining the rapidity of growth. Increased oxygen pressure was not used, as this is apparently unfavorable according to Moore and Williams.[9] Unless otherwise mentioned the inoculations were made from +1 glycerin agar cultures.

---

[1] *La salud. publ.*, 1907, 3, p. 34. References marked with asterisk were not consulted in the original.

[2] *Arch. f. Hyg.*, 1893, 16, p. 238.

[3] *Ztschr. f. Biol.*, 1894, 30, p. 221.*

[4] *Centralbl. f. Bakteriol.*, I Orig., 1893, 14, p. 316.

[5] *Ztschr. f. Hyg.*, 1894, 18, p. 128.

[6] *Compt. rend. Soc. de biol.*, 1912, 72, p. 48.*

[7] *Compt. rend. Acad. d. sc.*, 1912, 155, p. 860.*

[8] *Compt. rend. Soc. de biol.*, 1913, 74, p. 272.*

[9] *Biochem. Jour.*, 1909, 4, p. 177.

The stock solution "A" of salts contained 0.1 per cent each of NaCl, KCl, $CaCl_2$, and $MgSo_4$. Solution "B" differed from the above in having 0.1 per cent $Na_2CO_3$ in place of NaCl. These were dissolved in redistilled water. The salts used throughout the experiments were Kahlbaum's C.P.

### I. EXPERIMENTS RENDERING THE CULTURE NON-ACID-PROOF.

Transplants were made from a +1 glycerin agar culture, containing both acid-proof and non-proof bacilli, upon slants of Musgrave and Clegg's ameba agar (−1)[1] and upon slants of 2 per cent agar dissolved in distilled water (without clearing with egg white and hence without ovomucoid). Transplants upon these media were made about every two days, and one series was kept at 24–28° C. and another at 37° C. The growth always remained dryish and granular and never showed any tendency to wrinkle nor to produce pigment. On agar only the bacteria appeared as minute coccoid or diplococcoid bodies or as short rods. On the ameba agar the rods were usually longer, thinner, and often segmented or beaded. Throughout this time the rods retained the stain in Gram's method. During the first four weeks some acid-proof bacilli could always be found on careful search, but after the fifth week the growths remained non-acid-proof. When grown for 10 days under these conditions, the growth remained uniformly non-acid-proof and retained the same morphology and staining characters when grown again for three days on +1 glycerin agar. When suspended in 0.85 per cent NaCl solution, these cultures were not killed in 15 nor 30 minutes at 55° C. but were killed by heating to 60° C. for 15 minutes.

Hence it may be concluded that when kept under constant conditions unfavorable to the development of the acid-proof quality, this culture becomes wholly non-acid-proof though it still retains the stain in Gram's method.

---

[1] −1 = rendered 1 per cent alkalin to phenolphthalein with N/1 NaOH; +1 = rendered 1 per cent acid, by the addition of N/1 NaOH.

## II. EXPERIMENTS YIELDING THE ACID-PROOF QUALITY.

### A. ACQUISITION OF ACID-PROOFNESS WHEN ACETATES ARE THE SOURCE OF CARBON AND NITROGEN.

### EXPERIMENT 5.

SOL. A+THE FOLLOWING SOURCES OF CARBON AND NITROGEN.

| | | Growth | Remarks |
|---|---|---|---|
| 1.......... | 0.5 per cent ammonium acetate | + | In 22 days degree of acid-proofness like No. 2 in 12 days. Numerous minute pellicles. |
| 2.......... | Like 1, + 0.2 per cent $KH_2PO_4$ | ++ | In 12 days majority rods long, thin, curved, and strongly acid-proof. 22 days, completely acid-proof; pellicles 1–4 cm. in diameter. |
| 3.......... | Like 2, + 5 per cent glycerin | – | |
| 4.......... | Like 2, + 0.2 per cent each of potassium acetate and calcium acetate | +<br>– | About half of rods are acid-proof. |
| 5.......... | Like 4, + 5 per cent glycerin | – | |
| 6.......... | Like 5, + 0.2 gm., $MgSO_4$ and 0.2 gm. NaCl | – | |

A portion of the acid-proof pellicle on 2 was transplanted to similar media made up with Solution A in the one case and Solution B in the other. That inoculated onto Solution A sank to the bottom and there was no proliferation; that on B proliferated very slowly but on the 20th day, a thick, white, wrinkled pellicle covered about 4 square centimeters of surface. However, this growth was apparently derived from the non-acid-proof bacilli transplanted along with the acid-proof, for the smears made on the 20th day showed long, thin, curved, non-acid-proof rods along with a few acid-proof. This pellicle on B was again examined on the 36th day. It had developed a yellowish color and was extremely coherent. Smears showed that the matrix of the pellicle was filled with acid-proof rods and large acid-proof coccoid bodies. No non-acid-proof bacilli were present and the acidfast ones resisted prolonged decolorization.

An experiment varying the quantities of ammonium acetate and $KH_2PO_4$ showed the results given in Experiment 10.

On the 19th day, Cultures 2, 4, and 5, were sub-cultured on to +1 glycerin agar. In 48 hours Culture 4 yielded a luxuriant white growth, but the surface remained smooth with a ground-glass appearance. Subcultures 2 and 5 showed no proliferation for 12 days, but on the 19th day scattered colonies were present.

EXPERIMENT 10.

| | | Growth | Remarks |
|---|---|---|---|
| A2 | (Like 5A2), i.e., + 0.5 per cent ammonium acetate + 0.2 per cent $KH_2PO_4$ | + | Reached maximum growth covering one-tenth of the surface on 8th day, white, wrinkled pellicle. Majority of rods non-acid-proof, few strongly proof. On 19th day matrix of pellicle filled with strongly acid-proof curved and straight rods. |
| A3 | + 0.5 per cent ammonium acetate + 0.5 per cent $KH_2PO_4$ | +<br>− | Contaminated by mold. |
| A4 | + 0.2 per cent ammonium acetate + 0.2 per cent $KH_2PO_4$ | +++ | Reached a maximum growth on 8th day—white, wrinkled pellicle covering three-fourths surface chiefly non-acid-proof, but some strongly acid-proof rods present. On 19th day all acid-proof rods gone—coherent pellicle composed of short coccoid rods, non-acid-proof even when in masses. |
| A5 | + 0.5 per cent ammonium acetate + 0.1 per cent $KH_2PO_4$ | ++ | Reached a maximum growth on 8th day—white, wrinkled pellicle covering one-fourth of surface area. Smears on 8th and 9th days like A2. |

The addition of 0.2 per cent $NH_4Cl$ to Solution B containing 0.2 per cent ammonium acetate (with or without 0.2 per cent $KH_2PO_4$) was unfavorable. When Solution B contained 0.2 per cent potassium acetate or calcium acetate or sodium acetate with 0.2 per cent $NH_4Cl$ as source of nitrogen and 0.2 per cent $KH_2PO_4$, growth appeared only in the sodium acetate culture and then very slowly. On the 11th day this culture showed the presence of many strongly acid-proof rods but the majority were non-acid-proof.

### B. EXPERIMENTS YIELDING ACID-PROOF CULTURES, THE RESULT OF GROWTH IN THE PRESENCE OF AMMONIUM SALTS AND PROPYL ALCOHOL, AND MANNITE.

The results of growth in the presence of $NH_4Cl$, $KH_2PO_4$, and methyl, ethyl, propyl, butyl, and amyl alcohols and mannite, have already been described[1] in detail. Briefly, the culture became strongly acid-proof only in the propyl alcohol and mannite cultures and here the rods showed one or more strongly acid-proof granules resembling the so-called "spores" of the tubercle bacillus. Many "free spores" were also present.

The following variation in the experiment was made in Experiment 12a.

---

[1] To appear in Centralbl. f. Bakteriol., 1913.

1. +0.2 per cent $NH_4Cl$+0.2 per cent $KH_2PO_4$+1 c.c. propyl alcohol. (Same as Exp. VI–13.)
2. +0.2 per cent $NH_4Cl$ +0.2 per cent $KH_2PO_4$ + 2 c.c. propyl alcohol.
3. +0.2 " " +0.2 " " + 3 c.c. " "
4. +0.2 " " +0.2 " $(NH_4)_2HPO_4$ +1 c.c. propyl alcohol.
5. +0.2 " ammon. sulfate +0.2 per cent $(NH_4)_2HPO_4$+1 c.c. propyl alcohol.
6. +0.2 per cent ammon. sulfate +0.2 per cent $KH_2PO_4$ +1 c.c. propyl alcohol.

Growth was extremely slow but most rapid in Nos. 4 and 5. In about one month the entire surface was covered with small separate pellicles or with a continuous wrinkled pellicle (4 and 5). In the case of No. 3, the growth covered only about one-tenth of the surface area. Smears at this time showed that over half of the rods present were completely acid-proof throughout the series, and that in each case numerous extracellular and intracellular spore-like bodies were present.

### C. EXPERIMENTS YIELDING ACID-PROOF CULTURES, THE RESULT OF GROWTH IN THE PRESENCE OF CERTAIN CARBOHYDRATES.

Again, as previously described,[1] a luxuriant growth of acid-proof bacilli, otherwise resembling the cultures in propyl alcohol and mannite, were obtained in the presence of $NH_4Cl$, $KH_2PO_4$ and levulose. With the same ammonium salt and phosphate the following carbohydrates served as sources of carbon in the following order: about equal to levulose stood glucose; only slightly less favorable, galactose. After two months the percentage of acidity to phenolphthalein varied in these cultures between 4 and 6 per cent. There was very slight proliferation where maltose, lactose, and inulin were the sources of carbon. In spite of the slight growth in these last three cultures, the reaction of the medium was changed, at least in the maltose and lactose cultures which, at the end of two months, were 6 and 5 per cent acid to phenolphthalein. Possibly the enzymes set free from the bacilli here gave rise to products unsuitable for the synthesis of fatty acids and higher alcohols.

## III. INFLUENCE OF PHOSPHATES ON THE FERMENTATION OF CARBOHYDRATES.

Experiments showed that this culture could not utilize dextrose, levulose, or galactose nor the other carbohydrates mentioned above, nor the alcohol mannite, when $NH_4Cl$ was the source of N, in the absence of $KH_2PO_4$. This is probably to be explained on the basis of the brilliant work of Harden and Young, who were the first to show that the action of yeast zymase depended on the formation of a phosphorus-containing intermediary body—coenzyme.

---

[1] *Loc. cit.*

Quite a series of phosphates was found to act quite as favorably as $KH_2PO_4$ in the fermentation of levulose but only a few were favorable to the production of acid-proof bacilli, as follows:

EXPERIMENT 14.

| | | Growth | Remarks |
|---|---|---|---|
| 1......... | $NH_4Cl$ 0.2 per cent, levulose 0.2 per cent (c.c.) | – | |
| 2......... | Like 1, + 0.2 per cent $KH_2PO_4$ | +++ | Surface almost covered by pellicles in 48 hrs., when about one-half the long thin rods are strongly acid-proof. Likewise on 11th day—the acid-proof rods are often club-shaped. (Contaminated by pennicilium.) |
| 3......... | Like 1, + 0.2 per cent potassium phosphate (dibasic) | +++ | Surface completely covered by wrinkled, white pellicle on 5th day, when the rods are shorter than in 2 and non-acid-proof. Long, thin, irregularly stained, non-acid-proof rods still present on 11th day. |
| 4......... | Like 1, + 0.2 per cent sodium ammonium phosphate | +++ | Like 3 on 5th day, short rods and diplococcoids present on 11th day. |
| 5......... | Like 4, but without $NH_4Cl$ | +++ | Like 4. |
| 6......... | Like 1, + 0.2 per cent sodium biphosphate (primary) | ++ | Long, thin, non-acid proof rods in 48 hrs. Growth covers about one-half surface. On 11th day over one-half of the rods are strongly acid-proof and show numerous extracellular and intracellular "spores." |
| 7......... | Like 1, + 0.2 per cent sodium phosphate (secondary) | +++ | Long, thin, non-acid-proof rods in 48 hrs. On 11th day about half of the rods are acid-proof but not strongly so. Minute, spore-like bodies present both in acid-proof and in many non-acid-proof rods. |
| 8......... | Like 1, + 0.2 per cent sodium phosphate (tertiary) | +++ | Like 4. |
| 9......... | Like 1, + 0.2 per cent sodium phosphate (meta) | – | |
| 10......... | Like 1, + 0.2 per cent calcium phosphate (primary) | – | |
| 11......... | Like 1, + 0.2 per cent calcium phosphate (secondary) | ++ | Growth like 6 in 48 hrs., but pellicles are thicker—short non-acid-proof rods; on 11th day morphology and staining like 6. Not as many "free spores" present. |
| 12......... | Like 1, + 0.2 per cent calcium phosphate (tertiary) | +++ | Like 6 and 11 but the rods are smaller and the "free spores" vary greatly in size and are very numerous. |
| 13......... | Like 1, + 0.2 per cent sodium pyrophosphate | +++ | Surface almost covered in 72 hours. On 7th day the rods are chiefly short and non-acid-proof, but many larger and longer strongly acid-proof rods are present and many of these contain one or more strongly acid-proof granules. |

It will be noted that in the above series acid-proof rods developed only in the presence of potassium dihydrogen phosphate, calcium phosphate (secondary and tertiary), sodium biphosphate (primary), sodium pyrophosphate and to a lesser degree in the presence of sodium phosphate (secondary). In the same solution (B) as above, containing 0.2 per cent each of ammonium acetate and glucose there was no growth, but luxuriant growth took place in the same mixture plus 0.2 per cent sodium pyrophosphate.

Further, a single experiment (15) showed that in Solution B containing 0.2 per cent and 0.4 per cent respectively of $KH_2PO_4$ and $KNO_3$ and 0.05 per cent levulose, there was luxuriant growth in 5 days, whereas no growth occurred in one month in a similar solution without the phosphate.

## IV. EXPERIMENTS ON THE CHOICE OF VARIOUS SOURCES OF CARBON AND NITROGEN.[1]

1. +0.2 per cent each of urea and $KH_2PO_4$.—In three days, surface one-half covered with minute white pellicles. The thinner periphery of these pellicles was composed of minute non-acid-proof diplococcoid bodies, while the thicker central portion showed short thick rods some of which were acid-proof on the sixth day, but later, all the rods became non-acid-fast though still retaining the stain by Gram's method.

2. +0.2 per cent of $KH_2PO_4$ and 0.005 per cent of Theobromin.—On the 5th day a thin white pellicle covered about one-sixth of the surface and on the 19th day the surface was almost covered with thin, white, scattered pellicles composed of non-acid-proof short rods and diplococcoids.

A similar culture with caffein yielded no growth.

3. +0.2 per cent $KH_2PO_4$+0.2 c.c. acetone (Mercks technical).—On the 5th day a hazy growth covered one-eighth of the surface; on the 19th day small scattered pellicles almost covered the surface. These were composed of short non-acid-proof rods often showing a granule at each end or three granules.

4. The following combinations yielded no growth: (*a*) 0.2 per cent $KH_2PO_4$ with the separate addition of 0.2 per cent of ammonium carbonate and ammonium valerianate; (*b*) 0.2 per cent of

---

[1] Unless otherwise mentioned, Solution B was used.

ammonium bitartrate with or without 0.2 per cent $KH_2PO_4$; (*c*) 0.2 per cent each of $NH_4Cl$ and calcium lactate; (*d*) 0.2 per cent each of sodium formate, $NH_4Cl$, and $KH_2PO_4$; (*e*) Same as (*d*) but with 9 drops of formic acid in place of the formate without neutralization (reaction+1.2).

5. +0.2 per cent $NH_4Cl$+0.01 per cent leucin, tyrosin, alanin, glycocoll, aspartic acid, and glutamic acid.—No proliferation in presence of tyrosin, aspartic, and glutamic acids; extremely slight in glycocoll; fair proliferation on 4th day in presence of alanin and leucin with apparent preference for alanin. On the 9th day, the alanin culture was completely covered by a thin, white, veil-like skin studded by small, yellowish masses of growth (which probably represented the inoculated fragments) and the skin extended 3-4 mm. up the sides of the bottle. The rods were long, thin, non-acid-proof, and showed a very much segmented protoplasm. In addition there were numerous coccoid bodies about one-half of which were strongly acid-proof. The findings were similar on the 24th day, but now the culture was completely non-acid-proof.

In the more delicate culture in the presence of leucin, the bacteria appeared as very minute coccoid bodies about 0.5 $\mu$ in diameter and about half of these remained acid-proof up to the 24th day. In another experiment when, along with 0.2 per cent glycocoll, various sources of N were employed in Solution A, there was fair growth in 14 days in the following order: in the presence of 0.2 per cent each of—$KH_2PO_4$ and $(NH_4)_2SO_4$; $KH_2PO_4$ and $NH_4Cl$; $(NH_4)_2H.PO_4$ and $(NH_4)_2SO_4$; and $(NH_4)_2H.PO_4$ alone. Growth in these cultures appeared as very small thin scattered pellicles composed of non-acid-proof coccoid bodies and thin segmented rods. There was no growth in the following cultures: $NH_4Cl$ without $KH_2PO_4$, $KH_2PO_4$ and $KNO_3$, $KH_2PO_4$ and $KNO_2$, $(NH_4)_2SO_4$ without $KH_2PO_4$.

## CONCLUSIONS.

It seems worth while mentioning that when transplants were made from the cultures which had acquired acid-proofness in the presence of levulose, they showed little tendency to proliferate on +1 glycerin agar slants, but with few exceptions grew luxuriantly on +1 glucose agar slants. However, the resulting growths were non-acid-proof and the bacteria were of the coccoid type.

All attempts to show that the cultures in propyl alcohol and levulose—showing intracellular and free spore-like bodies—had a greater power to resist moist heat failed. All such cultures, when suspended in 0.85 per cent salt solution, were killed by heating at 60° C. for 30 minutes, though they were not killed by heating at 55° C. for 15 minutes. In a few instances, e.g., Culture Experiment XIV—6, no growth could be obtained on glycerin agar, glucose agar, nor on glycerin broth, when the subcultures were made from the unheated culture itself.

In the case of one heat test on Cultures XIV, 11 and 12, when the heated material failed to proliferate on glucose and glycerin agar, it was transferred to levulose agar slants. Still there was no growth.

However, it may be argued that the "spores" require special conditions for germination. Until the germination of these spore-like bodies has been actually seen, it may be well to consider their presence as due to especially rapid fat synthesis in one portion of the bacterial cell.

As already detailed by me, these artificially produced acid-proof and "sporulating" tubercle bacilli are readily stained in the unfixed condition by a large number of basic dyes but are not stained by Scharlach R. Yet the cultures are of a distinctly greasy and oily character when spread on a clean slide. Wells and Corper[1] have demonstrated the presence of lipase in *B. tuberculosis*, and it is known that certain bacteria, when grown in the presence of fats, will acquire acid-proof qualities. It is hardly necessary to go into detail concerning what is known of the substances giving bacteria the acid-proof quality, as the literature has been reviewed by Ritchie[2] and Wills.[3] While only chemical analyses can settle the point, the writer believes that the results of vital staining show that in all probability there is a qualitative or quantitative difference in the fatty acid, lipoid, and neutral fat content of the cultures described above and that obtaining in virulent tubercle bacilli.

---

[1] *Jour. Infect. Dis.*, 1912, 11, p. 388.

[2] *Jour. Path. and Bacteriol.*, 1905, 10, p. 334.

[3] *Centralbl. f. Bakteriol.*, I Orig., 1912, 61, p. 37.

## SUMMARY.

During a study of the growth of a saprophytic culture of *B. tuberculosis* in various synthetic media, it was observed that the morphology varied from minute coccoid bodies to short or long, thick or thin, straight or curved rods which were or were not acid-proof according to the conditions of growth.

The culture could be rendered non-acid-proof by continual growth under conditions unfavorable to the synthesis of fats.

The culture could synthesize fatty bodies rendering it acid-proof when such substances as acetates were the source of carbon and nitrogen; or from various ammonium salts and propyl alcohol; or from $NH_4Cl$ and mannite; or from $NH_4Cl$ and levulose, or from glycerin and glucose in the presence of peptone.

Various carbohydrates and the alcohol mannite were not attacked in the absence of phosphates. Various phosphates other than $KH_2PO_4$ favored fermentation, but acid-proof rods developed only in cultures containing $NH_4Cl$ and levulose in the presence of potassium dihydrogen phosphate, calcium phosphate (secondary and tertiary), sodium biphosphate (primary), sodium pyrophosphate, and to a lesser degree in the presence of sodium phosphate (secondary).

Under the conditions of the experiments the sporelike bodies produced were killed by heating to 60° C. for 30 minutes. That is, they were killed unless they require some very special conditions for germination.

From the results of vital staining it seems reasonable to conclude that the chemical composition of these artificially produced acid-proof tubercle bacilli is different from that of virulent tubercle bacilli.